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Effects of Anticancer Drugs, Metals and Antioxidants on Cytotoxic Activity of Benzothiepins/Benzoxepins

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Abstract. *Among 11 benzothiepins/benzoxepins, 4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5-(2H)-one [1] showed the highest cytotoxicity against human oral squamous cell carcinoma HSC-2 cells, followed by 2,3-dihydro-2-(2-oxopropyl)-2-phenyl-1-benzoxepin [2]. Popular antioxidants, such as N-acetyl-L-cysteine and sodium ascorbate significantly reduced the cytotoxic activity of [1] but not that of [2[. Compound [1] induced internucleosomol DNA fragmentation in human promyelocytic leukemic HL-60 cell line, but produced large DNA fragmentation in human oral tumor cell lines (HSC-2, HSG). Compounds [1] and doxorubicin additively reduced the viable cell number of HSC-2 cells. These data, taken together with their tumor specific action, demonstrate for the first time, the medicinal efficacy of benzothiepins/benzoxepins.*

We have newly synthesized 11 benzothiepins and benzoxepins, by cleavage of cyclopropane. Their medicinal significance was not known. We have recently found that most of these compounds showed higher cytotoxic activity against human oral tumor cells (HSC-2, HSG) than against normal cells such as human gingival fibroblast HGF (1). Among these compounds, 4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5-(2*H*)-one [1] (structure shown in Figure 1) showed the highest cytotoxicity, followed by 2,3-dihydro-2-(2-oxopropyl)-2-phenyl-1-benzoxepin [2]. The mechanism by which these compounds induced the cytotoxicity is unclear. We investigated here whether compound [1] can induce internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis (2), using various target cell lines. To test the possible involvement of oxidative stress, we also investigated the effect of several metals and antioxidants, on the cytotoxicity induced by compounds (1) and (2).

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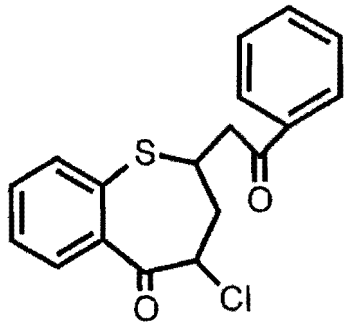
Materials and Methods

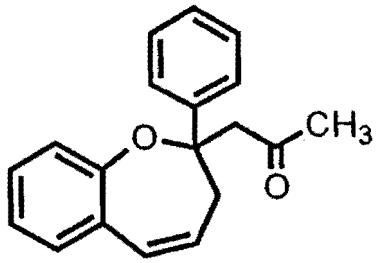
Materials. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Gland Island, NY, USA); fetal bovine serum (FBS) (JRH Biosic, Lenexa, KS, USA); $CuCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind. Ltd., Osaka, Japan); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), catalase, N-acetyl-L-cysteine (NAC) (Sigma Chem. Ind. St. Louis, MO, USA); RNase A, proteinase K (Boehringer Mannheim, Germany); doxorubicin (Kyowa-Hakko Ltd. Co., Tokyo, Japan).

Compounds [1] (4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5-(2H)-one) was synthesized as follows: to a stirred solution of 7a-chloro-1a, 7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (105 mg, 0.5 mmol) and 1-phenyl-1-trimethylsilyloxyethylene (192 mg, 1.0 mmol) in MeCN (4 mL) was added dropwise a solution of TMSOTf (33 mg, 0.15 mmol) in MeCN (0.5 mL) at 0°C under argon atmosphere. After being stirred for 30 minutes, the reaction was quenched at the same temperature by adding saturated aqueous $NaHCO_3$ (2 mL). The mixture was stirred vigorously for 10 minutes and allowed to warm to room temperature. The mixture was extracted with CH_2Cl_2 (20 mL x 3), the combined organic layers were dried over Na_2SO_4, then the solvent was evaporated under reduced pressure. The residue was dissolved in THF-1N HCl (2: 1, 6 mL) and the solution was stirred for 1 hour at 0°C. The mixture was extracted with ether (20 mL x 3), the combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: hexane-AcOEt = 20 : 1) to give the benzothiepin [1] (135 mg, 82%) (3).

Compound [2] (2,3-dihydro-2-(2-oxopropyl)-2-phenyl-1-benzoxepin) was synthesized as follows: to a stirred solution of isopropenyloxytrimethylsilane (130 mg, 1 mmol) and TMSOTf (11 mg, 0.05 mmol) in MeCN (4 mL) was dropwise added a solution of 1,1a,7,7a-tetrahydro-1a-phenylbenzo[*b*]cyclopropa[*e*]pyran-7-ol acetate (140 mg, 0.5 mmol) in MeCN (1 mL) over a 30 minute period at -40°C under an argon atmosphere. After being stirred for 30 minutes, the reaction was quenched at the same temperature by adding saturated aqueous $NaHCO_3$ (2 mL). The mixture was vigorously stirred for 10 minutes and allowed to warm to room temperature. The mixture was



4-chloro-3,4-dihydro-2-(2-oxo-2-phenyethyl)-1-benzothiepin-5-one [1]



2,3-dihydro-2-(2-oxopropyl)-2-phenyl-1-benzoxepin [2]

Figure 1. *Structure of 4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5-(2H)-one [1] and 2,3-dihydro-2-(2-oxopropyl)-2-phenyl-1-benzoxepin [2].*

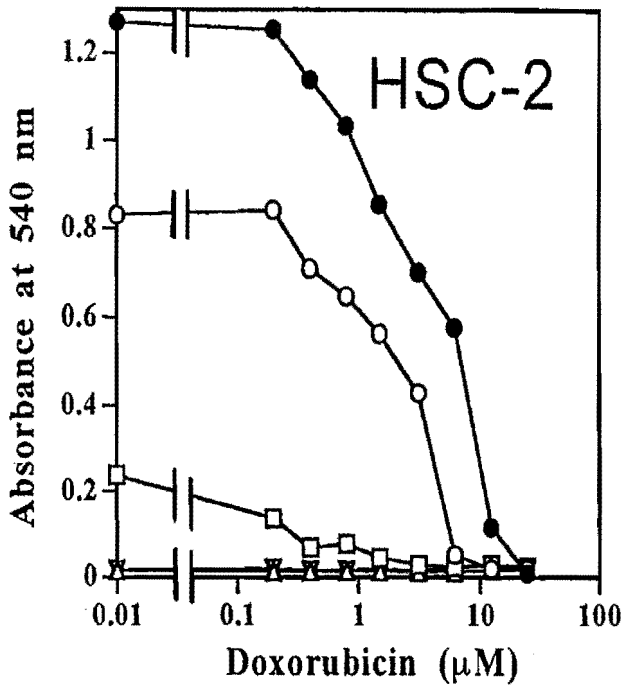


Figure 2. *Combination effect of doxorubicin and 4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5-(2H)-one [1]. Near confluent HSC-2 cells were incubated for 24 hours with the indicated concentrations of doxorubicin in the presence of 0(●), 12.5(○), 25(□), 50(△) or 100(▽) μg/ml compound [1] and the relative viable cell number (A_{540}) was then determined by MTT method.*

extracted with CH_2Cl_2 (20 mL x 3), the combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: hexane-AcOEt=30:1) to give the benzoxepin (2) (97 mg, 70 %) (3).

Cell culture. Human oral squamous cell carcinoma HSC-2 and human salivary gland tumor (HSG) cells were cultured in DMEM medium supplemented with 10% FBS in a humidified 5% CO_2 atmosphere. Human promyelocytic leukemia HL-60 cells were cultured in RPMI1640 medium supplemented with 10% FBS.

Assay for cytotoxic activity. Near confluent HSC-2 cells grown in 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickenson) were incubated for 24 hours with various concentrations of samples. The cells were washed with phosphate-buffered saline and incubated for 4 hours with fresh culture medium containing 0.2 mg/ml MTT. After removing the medium, the cells were lysed with 100 μl DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate with Labsystem Multiska® (Biochromatic) with Star/DOT Matrix Printer JL-10. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Table I. *Effect of metals and antioxidants on the cytotoxic activity of benzothiepinone, and benzoxepin against HSC-2 cells.*

	Cytotoxic activity (CC_{50}: μg/ml)	
Addition	Compound [1]	Compound [2]
None (control)	11	45
+ 0.2 mM $CuCl_2$	8	42
+ 0.2 mM $FeCl_3$	11	45
+ 3000 unit/ml catalase	13	40
+ 4 mM NAC	28	45
+ 0.25 mM sodium ascorbate	20	43

Near confluent HS-2 cells were inculated for 24 hours with various concentrations of compound [1] or [2] in the absence (control) or presence of the indicated concentrations of metal or antioxidant. Control A_{540} of HSC-2 cells were 0.826. Each value represents mean from 2-3 determinations.

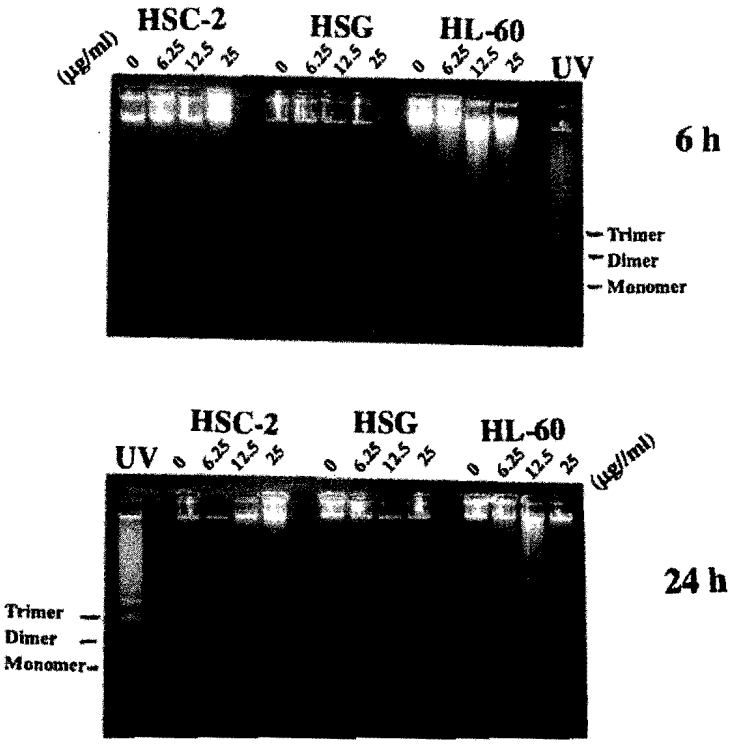


Figure 3. *Induction of DNA fragmentation by compound [1]. Near confluent HSC-2, HSG or HL-60 cells were incubated for 6 or 24 hours with the indicated concentrations (μg/ml) of compound [1] and the DNA fragmentation was assayed by agarose gel electrophoresis. UV represents DNA from apoptotic HL-60 cells which were incubated for 6 hours in culture medium after exposure to UV irradiation (6J/m^2/minute, 1 minute).*

Assay for DNA fragmentation. The cells were pelleted, lysed and digested with RNase A and proteinase K. DNA was isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (4).

Results and Discussion

When human oral squamous cell carcinoma HSC-2 cells were incubated with benzothiepinone [1] or benzoxepin [2], the viable cell number was dose-dependently reduced (Figure 2, Table I). The 50% cytotoxic concentration (CC_{50}) of these compounds was 11 and 45 μg/ml, respectively. The cytotoxic activity of compound [1] was significantly reduced by NAC or sodium ascorbate, suggesting the possible involvement of active oxygen species (Table I). Hydrogen peroxide might not be involved, since catalase and metals (Cu^{2+}, Fe^{3+}) did not affect the cytotoxic activity of compound [1]. On the other hand, the cytotoxic activity of compound [2] was not significantly affected by any of these agents (Table I).

When HL-60 cells were incubated for 6 or 24 hours with 12.5 μg/ml compound [1], internucleosomal DNA fragmentation was induced (Figure 3). However, higher or lower concentrations of compound [1] did not induce the DNA fragmentation, indicating that the optimum concentration of [1] was very narrow. In contrast, compound [1] did not induce internucleosomal DNA fragmentation, but produced large DNA fragments in HSC-2 or HSG cells (Figure 3). Similarly, ascorbic acid, hydrogen peroxide, etoposide, tumor necrosis factor, hyperthermia and UV irradiation induced internucleosomal DNA fragmentation only in human myelogenous leukemic cell lines (HL-60, ML-1, U-937, THP-1), but not in other cell lines including T-cell leukemia (MOLT-4) or erythroleukemia (K-562) cell lines (4). Furthermore, geranylgeraniol and vitamin K_2 derivatives induced DNA fragmentation in HL-60 cells, but not in HSC-2 or HSG cell lines (5). These data suggested that whether apoptosis-inducing agents induce internucleosomal DNA fragmentation or produce large DNA fragments depends upon the target cells. This raises the question as to whether internucleosomal DNA fragmentation is truly universal hallmark of apoptosis (6).

When HSC-2 cells were incubated with increasing concentrations of compound [1] and doxorubicin (a popular anticancer agent), their cytotoxic activity was additively enhanced (Figure 2). The present study, taken together with its tumor specific action (1), demonstrated

for the first time the possible medicinal efficacy of compound [1]. The mechanism by which this compound induces cell death in cancer cell lines remains to be investigated.

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